Synthetic furanones and inhibition of biofilm formation

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Jessica Lönn-Stensrud

Summary

In this study we investigated whether furanones could prevent biofilms from being formed. Our first aim was to find a new synthesis pathway for (*Z*)-5-bromomethylene-2(5*H*)-furanone, a furanone also produced in nature by the macro-alga *Delisa pulchra*, and to investigate its effects on biofilm formation by *Streptococcus anginosus*, *Streptococcus intermedius*, *Streptococcus mutans* and *Staphylococcus epidermidis*. We also synthesised 5-(bromomethylene)furan-2(5*H*)-ones and 3-(bromomethylene)isobenzofuran-1(3*H*)-ones to further investigate their effects on biofilm formation by *S. epidermidis.* The effect on biofilm formation was assessed either by adding furanone to the growth medium or by allowing biofilm to form on surface-adsorbed furanone. Furanone effectively decreased biofilm formation by both application methods, but with surface-adsorbed furanone being most effective. The furanone concentrations used had no effect on microbial growth, and were also well below the established MICs, indicating that the effect was not a result of an antimicrobial effect. In order to determine whether the inhibitory effect was associated with the ability of furanone to interfere with microbial AI-2 communication, AI-2 deficient mutants were included. In contrast to their wild types, the amount of biofilm formed by the communication defective *S. anginosus* and *S. intermedius* was not affected when adding furanone to the growth medium. Biofilm formation on surface-adsorbed furanone was unaffected or partially affected for the communication defective *S. anginosus* and *S. intermedius* mutants respectively. Adding DPD, the synthetic AI-2 communication signal, to the growth medium abolished the inhibitory effect of furanone on biofilm formation. Furthermore furanones were found to decrease bioluminescence by the AI-2 reporter strain. Taken together, these findings support the assumption that furanones interfere with microbial communication, without affecting microbial growth.

Finally, we studied possible irritative and genotoxic effects of the furanones. Only after using 1000 times higher concentrations than used in the biofilm assay, did we see an irritative effect. There were no significant alterations of the genotoxic and global gene expression in mice treated with furanone.

Interference with microbial communication thus may represent a novel and promising strategy to control biofilm related infections.

Sammandrag

I denna studien har vi undersökt om furanoner kan förhindra bildandet av biofilmer. Vår första målsättning var att finna en ny syntesväg för (*Z*)-5-bromomethylene-2(5*H*)-furanone, en furanon som produceras naturligt av makro-algen *Delisa pulchra*, samt att undersöka dess effekt på biofilmer av *Streptococcus anginosus*, *Streptococcus intermedius* och *Streptococcus mutans***.** Vi syntetiserade även 5-(bromomethylene)furan-2(5*H*)-ones och 3-(bromomethylene)isobenzofuran-1(3*H*)-ones för att vidare kunna undersöka deras effekt på biofilm av *Staphylococcus epidermidis.* Effekten på biofilm blev undersökt genom att tillsätta furanoner till växtmediet eller genom att observera hur biofilm bildas på en ytadsorberad furanon. Furanonerna minskade effektivt bildandet av biofilm i båda applikationsmetoderna, men ytadsorberad furanon var mest effektiv. Koncentrationerna av furanon som användes hade ingen effekt på bakteriell tillväxt och koncentrationerna låg också långt under de fastställda MIC (Minimal Inhibitory Concentration)-värdena, vilket indikerade att effekten inte var ett resultat av en antimikrobiell effekt. För att kunna bestämma om den inhibitoriska effekten var associerad med furanonernas förmåga att påverka mikrobiell AI-2 kommunikation, inkluderades en mutant som saknar förmåga att kommunicera genom AI-2. Till skillnad från motsvarande vildtyp var mängden biofilm bildad av *S. anginosus* and *S. intermedius* mutanterna, som saknar förmåga att kommunicera genom AI-2, inte påverkad av furanoner tillsatta till växtmediet. Mängd bildad biofilm av *S. anginosus* and *S. intermedius* mutanterna på ytadsorberad furanon var oförändrad eller endast delvis påverkad hos de respektive mutanterna. Genom att tillsätta DPD, den syntetiska AI-2 kommunikationssignalen, till växtmediet kunde den inhibitoriska effekten av furanon upphävas. Vidare fann vi att furanon minskade bioluminescence hos AI-2 reporter-stammen. Tillsammans understöder resultaten antagandet att furanoner påverkar mikrobiell kommunikation utan att påverka mikrobiell tillväxt.

Slutligen undersökte vi möjliga skadliga och genotoxiska effekter av furanoner. Först efter att ha använt 1000 gånger högre koncentrationer än de vi använde i biofilm-försöken såg vi en skadlig effekt. Vi såg inga signifikanta förändringar i våra genotoxiska tester hos möss som blivit behandlade med furanon.

Möjligheten att kunna påverka bakteriell kommunikation kan därför representera en ny och lovande strategi för att kunna kontrollera bakteriella biofilmrelaterade infektioner.

Publications included

This thesis is based on the following manuscripts, hereafter cited by their roman numerals:

I. Tore Benneche, Jessica Lönn, Anne Aamdal Scheie. 2006. Synthesis of (*E*)- and (*Z*)-5-(Bromomethylene)furan-2(5*H*)-one by Bromodecarboxylation of (*E*)-2-(5-Oxofuran-2(5*H*)-ylidene)acetic Acid. Synthetic communications. 36: 1401-1404

II. Jessica Lönn-Stensrud, Fernanda C. Petersen, Tore Benneche, Anne Aamdal Scheie. 2007. Synthetic bromated furanone inhibits autoinducer-2-mediated communication and biofilm formation in oral streptococci. Oral Microbiology and Immunology. 22: 340-346

III. Tore Benneche, Zainab Hussain, Anne Aamdal Scheie, Jessica Lönn-Stensrud. 2008. Synthesis of 5-(bromomethylene)furan-2(5*H*)-ones and 3-(bromomethylene)isobenzofuran-1(3*H*)-ones as inhibitors of microbial quorum sensing. New J. Chem. 32: 1567-1572

IV. Jessica Lönn-Stensrud, Maria A. Landin, Fernanda C. Petersen, Tore Benneche, Anne Aamdal Scheie. 2009. Furanones, potential agents for preventing *Staphylococcus epidermidis* biofilm infections? J. Antimicrob. Chemother. 63: 309 - 316

Abbreviations

AHL	acylated homoserine lactone
AI	Autoinducer
AI-2	Autoinducer-2
DPD	(*S*)-4,5-Dihydroxy-2,3-pentanedione
	(*S*)-4,5-Dihydroxypentane-2,3-dione
MIC	Minimum inhibitory concentration
TFA	Trifluoroacetic acid
F101	(*Z*)-5-(Bromomethylene)dihydrofuran-(3*H*)-one
F201	(*Z*)-5-(Chloromethylene)furan-2(5*H*)-one
F202	(*Z*)-5-(Bromomethylene)furan-2(5*H*)-one;
	(*Z*)-5-Bromomethylene-2(5*H*)-furanone
F203	(*Z*)-5-(Bromomethylene)-3-methylfuran-2(5*H*)-one
F204	(*Z*)-5-(Bromomethylene)-3,4-dimethylfuran-2(5*H*)-one
F205	(*E*)-5-(Bromomethylene)-3,4-dimethylfuran-2(5*H*)-one
F206	(*Z*)-3-Bromo-5-(bromomethylene)furan-2(5*H*)-one
F207	(*E*)-5-(Bromomethylene)-3-phenylfuran-2(5*H*)-one
F208	(*Z*)-5-(Bromomethylene)-3-phenylfuran-2(5*H*)-one
F301	(*E*)-3-(Bromomethylene)isobenzofuran-1(3*H*)-one
F302	(*Z*)-3-(Bromomethylene)isobenzofuran-1(3*H*)-one

Introduction

Biofilm and biofilm formation

Microorganisms were long thought of as floating single cells, without the ability to interact with or react to their environment or surrounding microorganisms. During the last two decades, it has become clear that the preferred growth mode of microorganisms is in biofilms. Microorganisms in biofilms live in well-organised communities consisting of single or multiple species embedded in an organic polymer matrix adhering to a surface (Costerton *et al.*, 1995). The first description of microbial biofilms was made as early as in the 1930s (ZoBell and Allen, 1933; ZoBell and Allen, 1935). Later microbial biofilms were found to be ubiquitous, as they were found in vastly different aquatic ecosystems in nature, in industry, and in medicine (Costerton *et al.*, 1978; Costerton *et al.*, 1995). The biofilm provides improved survival ability for the microorganisms through attachment to surfaces, as well as genetic heterogeneity. This may be seen as a specialisation of function, resembling the division of function seen in multicellular organisms (Jefferson, 2004).

The process of biofilm formation is thought to be well regulated (Lazazzera, 2005). The process starts with the adhesion of planktonic microorganisms to a surface, followed by colonisation and co-adhesion, growth and maturation, and finally detachment of some microorganisms (Scheie and Petersen, 2004). At the first stage, the microorganisms are reversibly attached, and not yet committed to the biofilm differentiation process. To develop a mature biofilm, the adhesion must be irreversible and the microorganisms must grow in population. There are three mechanisms that may lead to colonisation and co-adhesion; redistribution of attached cells by surface motility, division of attached cells, or recruitment of planktonic cells to the developing biofilm. The biofilm formation is a dynamic process, thus attachment, co-adhesion and growth may occur at overlapping times. Which mechanism will be the most predominant depends on the organism involved, the nature of the surface being colonised, and the physical and chemical conditions of the environment. The maturation of the biofilm results in a complex architecture with channels and pores. The microorganisms, individually or in groups, may also be released from the biofilm surface to find other colonisation sites. It has been hypothesised that the released microorganisms have more similarity to planktonic microorganisms than to biofilm microorganisms, thereby closing the biofilm lifecycle (Stoodley *et al.*, 2002).

Biofilms associated with infectious diseases

All body fluids provide organic nutrients for microbial growth, and body surfaces may harbour various combinations of microorganisms, living in an ecological equilibrium with the host. In a commensal ecosystem within the body, inhabiting microorganisms may be a protection to the host. In case the balance shift to the advantage of specific microorganisms, diseases or infections may develop. There is a direct and dynamic relationship between an environment and its microflora, even at the microhabitat scale. Substantial changes to a habitat may cause an imbalance between the resident organisms (Costerton *et al.*, 1995; Marsh, 2003).

Within the microbial ecosystem of the oral cavity, we find a number of distinct surfaces with different key ecological factors. These factors enable some organisms to dominate at one site, whilst others dominate at others. Such distinct habitats include lips, cheek, palate, tongue, and teeth. Disruption of the balance at these sites may lead to dental caries or periodontal disease (Marsh, 2003).

The oral cavity is an ecosystem easily accessible for research, making it a suitable model for biofilm studies. Several *in vitro* models mimicking this oral ecosystem have been applied. Results from these models have revealed the interactions and activity of microorganisms in the oral cavity, as well as contributed to the understanding of biofilms in general (Kolenbrander *et al.*, 2002). Depending on the microbial composition, biofilm formation may be conducive with health, or lead to disease (Marsh, 2003). Biofilm formation may be critical for the pathogenesis (Costerton *et al.*, 1999).

Oral streptococci are commensals of the oral cavity and upper respiratory tract of humans and animals (Whiley and Beighton, 1998). They are primary colonisers, and as such mediate subsequent attachment of other microorganisms to form a mature oral biofilm (Nyvad and Kilian, 1987). Although they are commensals, oral streptococci are among the species that may be isolated from biofilm-infected medical devices (Donlan, 2001). Oral streptococci also account for half of all the cases of infective endocarditis (Costerton *et al.*, 1999).

The anginosus group, including *Streptococcus anginosus*, *Streptococcus intermedius*, and *Streptococcus constellatus*, are commensally present in saliva and gingival plaque (Whiley and Beighton, 1991; Whiley *et al.*, 1993; Claridge *et al.*, 2001) as well as in the gastrointestinal and the genito-urinal tracts (Piscitelli *et al.*, 1992; Whiley *et al.*, 1992). Members of the anginosus group have also been associated with infectious diseases. Both *S. anginosus* and *S. intermedius* are commonly isolated from endodontic infections in the root

canals (Gomes *et al.*, 2004; Chavez de Paz *et al.*, 2005), whilst *S. intermedius* has been isolated from periodontal disease (Haffajee *et al.*, 1988), and implantitis (Tanner *et al.*, 1997). Members of the anginosus group have also been found to colonise dental implant surfaces and may be the cause of early implant failure (Laine *et al.*, 2005). The anginosus group may also be associated with septicaemia, as well as abscesses in for instance gastrointestinal and genito-urinal tracts, and the central nervous system (Whiley *et al.*, 1992; Clarridge *et al.*, 1999; Claridge *et al.*, 2001).

Streptococcus mutans is also a commensal of the oral flora. Preferentially, *S. mutans* colonises the tooth surface, and has also been found to colonise prosthetic devices. Their ability to adhere to smooth surfaces, as well as their acidogenic properties, are virulence factors associated with dental caries development. Glucan-producing streptococci, including *S. mutans*, has also been connected with sub-acute endocarditis (Hamada and Slade, 1980).

Staphylococcus epidermidis makes up a major part of the normal flora of human skin and was long considered to be a harmless commensal. However, in the past decades, *S. epidermidis* has emerged as a major pathogen in biofilm infections associated with medical devices (Hussain *et al.*, 1993). The first observation of staphylococcal colonisation of medical devices was made in 1961 and was referred to as slime production. This slime production was thought of as a special feature for this particular strain (Callaghan *et al.*, 1961). First in 1982, Christensen and co-workers realised that slime production could be an overlooked pathogenic characteristic of many *S. epidermidis* strains (Christensen *et al.*, 1982).

The increased use of implanted medical devices, as well as the increased use of antimicrobials, have turned *S. epidermidis* to one of the major causes of nosomical infections. Today, infections by *S. epidermidis* are associated with a broad spectrum of medical devices, such as vascular catheters (Raad and Bodey, 1992; Rupp and Archer, 1994), cerebrospinal shunts (van Ek *et al.*, 1986), and prosthetic joints (Inman *et al.*, 1984). The increasing use of antimicrobials to prevent and treat infections in hospitalised patients have led to the emergence of multi resistant *S. epidermidis* (Raad *et al.*, 1998).

Microbial communication

Microorganisms respond to their fellow habitants and sense the surrounding environmental conditions through different mechanisms. One way to achieve this is through a process where the microorganisms react to signalling molecules by altering their gene expression in a density dependent manner, so called quorum sensing.

Cell-cell communication was described already in the 1960s. Pakula and Walczak described an exocellular factor that provoked competence in streptococci under certain conditions and upon addition to non-competent cultures (Pakula and Walczak, 1963). In 1964, Kerravala and co-workers demonstrated that *Bacillus subtilis* and *Bacillus cereus* strains produced an endogenous factor that initiated sporogenesis in vegetative cells not yet committed to sporulation. The initiation of sporogenesis occurred at specific cell densities (Kerravala *et al.*, 1964). Shortly after, data indicated that the competent state in "*Pneumococcus*" (later *Streptococcus pneumoniae*), was regulated by an extracellular activator. This behaviour was described as an environmental sensing mechanism which was termed autoinduction (Tomasz, 1965). Later, studies of the marine light producing microorganism *Vibrio fischeri* revealed that light emission was induced by a signalling molecule, termed autoinducer (AI). As this AI accumulated, the microorganisms responded by synthesizing luminescence enzymes (Nealson *et al.*, 1970; Nealson, 1977). In the 1980s, a model for autoinduction in *V. fischeri* was suggested. This model indicated that the transcription of different genes was controlled by a threshold AI concentration. The AIs were suggested to be produced intracellularly, diffusing to the outside, and thereby being able to signal to neighbouring microorganisms (Engebrecht *et al.*, 1983; Kaplan and Greenberg, 1985). Quorum sensing allows microorganisms to regulate genes according to cell density, thereby inducing a behavioural response throughout the whole population. Genes controlled by quorum sensing are connected to behaviour being productive only when carried out by many microorganisms simultaneously, e.g. secretion of virulence factors, biofilm formation, sporulation and bioluminescence (Miller and Bassler, 2001; Henke and Bassler, 2004).

The three most studied quorum sensing communication systems involve the LuxI/R system using acylated homoserine lactones (AHLs) as autoinducers for species specific communication in Gram negative microorganisms, the oligopeptide autoinducer system for species specific communication in Gram positive microorganisms, and an interspecies system used by both Gram negative and Gram positive microorganisms using a furanosyl borate di-ester termed autoinducer-2 (AI-2) as the signalling molecule (Henke and Bassler, 2004).

Autoinducer-2

The communication based on AI-2 was first described in *Vibrio harveyi* (Bassler *et al.*, 1994), and was later suggested to be a common intra- and inter-species communication system (Bassler *et al.*, 1997). AI-2 is a product of the LuxS enzyme (Schauder *et al.*, 2001; Xavier and Bassler, 2003), and a by-product of the metabolic activated methyl cycle that recycles S-

adenosylmethionine (Sun *et al.*, 2004). The LuxS coding gene is conserved in a majority of sequenced Gram-positive and Gram-negative microbial genomes (Chung *et al.*, 2001; Fong *et al.*, 2001; Frias *et al.*, 2001; Burgess *et al.*, 2002; Wen and Burne, 2002; McNab *et al.*, 2003; Merritt *et al.*, 2003; Vendeville *et al.*, 2005; Yoshida *et al.*, 2005; Petersen *et al.*, 2006), suggesting that AI-2 may function as a universal language for interspecies communication (Vendeville *et al.*, 2005).

Supernatants from oral streptococci, including *S. anginosus, S. intermedius,* and *S. mutans*, induced bioluminescence in the *V. harveyi* BB170 reporter sensing AI-2. This suggests that these streptococci produce an AI-2 like molecule for inter-species communication (Petersen *et al.*, 2006). LuxS is identified in various oral microorganisms, including *S. anginosus, S. intermedius,* and *S. mutans*, where AI-2 has been associated with virulence and biofilm formation (Merritt *et al.*, 2003; Wen and Burne, 2004; Yoshida *et al.*, 2005; Petersen *et al.*, 2006; Ahmed *et al.*, 2008).

LuxS has also been identified in *S. epidermidis*, suggesting that AI-2 has a signalling function in *S. epidermidis* (Xu *et al.*, 2006). However, the mechanism of AI-2, and data on the role in biofilm formation are inconclusive (Kong *et al.*, 2006).

Prevention and treatment of implant related biofilm infections

There is a close relationship between biofilms and infectious diseases, both for conditions such as cystic fibrosis and periodontitis, as well as infections in the bloodstream and urinary tract resulting from various indwelling medical devices (Donlan and Costerton, 2002).

Plastic and metallic surfaces of medical implants may accrete microorganisms dislodged from the body surfaces, thus leading to infection. Shear forces, such as blood flow or blinking of the eye when wearing contact lenses, is not sufficient to prevent microbial adhesion or biofilm formation. Once a biofilm is established on a medical device, the microorganisms are protected from host defence and antimicrobials (Costerton *et al.*, 1995). Biofilms may be up to 1000-fold more resistant than their planktonic counterparts (Gilbert *et al.*, 1997). Infections associated with biofilms on medical devices are extremely difficult to treat. This may be connected with restricted penetration of antimicrobials, decreased growth rate of biofilm cells, the expression of resistance genes, or the presence of persister cells (Costerton *et al.*, 1999).

Current approaches to control biofilm related implant infections concentrate on preventing initial device contamination and microbial attachment to the device, while treatment of an existing infection normally involves antimicrobial treatment and removal of

the device. Such approaches are, however not always effective in controlling biofilm infections and the risk of implant related microbial infections is still rather high (O'Gara and Humphreys, 2001; Donlan and Costerton, 2002).

A major concern is the development and spread of antimicrobial resistance. Infections once being easy to cure with antimicrobials are now becoming difficult, and sometimes even impossible, to treat due to multidrug resistance. In 2004, nearly 2 million people in the United States were assumed to acquire hospital associated microbial infections. More than 70 % of the microorganisms involved in these infections were thought to be resistant to at least one of the drugs used to fight them. Some of the resistant microorganisms, earlier found only in hospitals, are now found in the community, in otherwise healthy individuals. This gives an even greater risk of spreading resistant strains (Infectious Diseases Society of America, July, 2004).

Since few effective means to prevent medical device-related infections are currently available, research is called for. The antimicrobials used today were developed and generally tested against planktonic microorganisms in the laboratory, disregarding the increased resistance against antimicrobials in microorganisms living in biofilms (Smith, 2005). It is therefore necessary to look for new approaches to combat biofilm infections. Controlling microbial biofilm formation by targeting microbial communication could be a novel alternative. Such an approach would not kill the microorganisms, nor exert a selective pressure on the microorganisms. This would in best-case scenario not lead to resistance development. Halogenated furanones provide an interesting potential.

Furanones - a new approach

The discovery that the alga *Delisea pulchra*, in contrast to other algae in the surroundings, remained free from colonising organisms, challenged researchers to find an explanation. Studies showed that the alga produced various structurally similar halogenated furanones as secondary metabolites (Kazlauskas *et al.*, 1977; Pettus *et al.*, 1977; de Nys *et al.*, 1992; de Nys *et al.*, 1993; de Nys *et al.*, 1996; de Nys *et al.*, 1998). The furanones were found to be encapsulated and delivered to the surface of the alga at concentrations deterring both prokaryote and eukaryote fouling organisms. The inhibitory effect was assumed to be mediated through interference with microbial communication (Dworjanyn *et al.*, 1999; Kjelleberg and Steinberg, 2001). Crude extracts of furanones isolated from the alga were also shown to be effective against early colonising marine microorganisms by repressing swarming, and by partly repressing attachment and growth (Maximilien *et al.*, 1998).

Furanones from *Delisea pulchra* were shown to specifically interfere with a well conserved gene regulatory system found in a wide range of Gram-negative microorganisms (Givskov *et al.*, 1996; Kjelleberg *et al.*, 1997; Manefield *et al.*, 1999; Manefield *et al.*, 2002). This gene regulatory system involves a small diffusible signal molecule, AHL, also known as AI-1, which accumulates in the environment and thereby up- or down regulates specific genes according to cell-density in the surrounding (Fuqua *et al.*, 1996). Later it was found that natural bromated furanones isolated from the alga, as well as their synthetic derivatives, inhibit multi-cellular behaviour induced by AI-1 such as swarming (Givskov *et al.*, 1996; Gram *et al.*, 1996; Rasmussen *et al.*, 2000), biofilm formation (Hentzer *et al.*, 2002; Hentzer *et al.*, 2003; Wu *et al.*, 2004) expression of virulence factors (Hentzer *et al.*, 2003), as well as affecting the synthesis of siderophores (Ren *et al.*, 2005), without affecting growth.

Natural furanones isolated from *Delisea pulchra* were also found to inhibit swarming and biofilm formation induced by the universal AI-2 signal molecule used by Gram negative microorganisms (Ren *et al.*, 2001) without affecting growth. The furanones also repressed the expression of several AI-2 induced genes connected to chemotaxis, motility and flagella (Ren *et al.*, 2004). Furanones have also been shown to decrease swarming and biofilm formation by *Bacillus subtilis* and virulence gene expression by *Bacillus anthracis*, but with an effect on growth (Ren *et al.*, 2002; Jones *et al.*, 2005). A later study showed that furanones induced genes closely connected to stress response in *Bacillus subtilis*, as well as fatty acid synthesis, ribose transport and metabolism amongst others, whereas general growth genes were not affected (Ren *et al.*, 2004).

Synthetic furanones similar to the natural furanones isolated from *Delisea pulchra* were shown to inhibit microbial communication mediated by AI-1 (Hentzer *et al.*, 2002; Manefield *et al.*, 2002; Wu *et al.*, 2004). At the start of the present study, there were a few studies on the effect of furanones on AI-2 mediated microbial communication in *Escherichia coli* (Ren *et al.*, 2001; Ren *et al.*, 2004), but no studies on a possible furanone effect on Gram positive microorganisms. Since several Gram-positive microorganisms regulate biofilm formation via AI-2 communication (Schauder *et al.*, 2001; Federle and Bassler, 2003; McNab *et al.*, 2003; Merritt *et al.*, 2003; Yoshida *et al.*, 2005; Petersen *et al.*, 2006; Rickard *et al.*, 2006), our hypothesis was that synthetic furanone would inhibit biofilm formation via AI-2 communication interference in these microorganisms. We were thus challenged to synthesise furanones and to assess possible effects on AI-2 dependent biofilm formation, in several Gram-positive microorganisms. It was furthermore intriguing to consider furanones' suitability regarding irritability and toxicity.

Aims of the thesis

The major aim was to synthesise chemically distinct furanones and to assess their ability to interfere with biofilm formation, as well as their irritative and toxic effects.

The specific aims were to:

1. Find alternative ways to synthesise (*Z*)-5-(bromomethylene)furan-2(5*H*)-one (Paper I) as well as different 5-(bromomethylene)furan-(5*H*)-ones and 3-(bromomethylene)isobenzofuran-1(3*H*)-ones (Paper III).
2. Investigate the effect of furanones on *S. anginosus*, *S. intermedius*, *S. mutans* (Paper II), and *S. epidermidis* biofilm formation (Paper III, Paper IV).
3. Elucidate the mechanisms of action of the furanones by investigating
    - the furanones antimicrobial activity (Paper II, Paper III, Paper IV).
    - the ability of the synthetic furanones to interfere with AI-2 communication assessed as bioluminescence inhibition (Paper II, Paper III, Paper IV).
    - the furanones effect on biofilm formation by *luxS* mutants compared to their wild types (Paper II).
    - whether the effect could be reversed by (*S*)-4,5-dihydroxy-2,3-pentanedione (DPD), the precursor of AI-2 (Paper IV).
4. Assess possible irritative and genotoxic effects (Paper IV).

Summary of results

Furanone synthesis

A new, easy and highly productive way to synthesise (*Z*)-5-bromomethylene-2(5*H*)-furanone (F202), a furanone found in the alga *Delisa pulchra*, was established. (*Z*)-5-bromomethylene-2(5*H*)-furanone (F202) was synthesized in four steps from the commercially available adduct between furan and a maleic anhydride. A reaction between the derivative of the two starting products and a phosphorane gave the ester (*E*)-tert-butyl 2-(5-oxofuran-2(5*H*)-ylidene)acetate. The ester was then treated with trifluoroacetic acid to form the acid (*E*)-2-(5- oxofuran-2(5*H*)-ylidene)acetic acid. Finally the (*Z*)-5-(bromomethylene)furan-2(5*H*)-one was prepared by bromodecarboxylation of the acid. After synthesis, the furanone was dissolved and diluted in absolute ethanol to a final stock concentration of 60 mM and stored at -20^{o} C until use (Paper I).

To further investigate possible effects of furanones, 5-(bromomethylene)furan-2(5*H*)-ones and 3-(bromomethylene)isobenzofuran-1(3*H*)-ones were synthesised and thereafter assessed for their ability to interfere with microbial communication.

The furanones were synthesised in three or four steps from commercially available maleic anhydrides and phthalic anhydrides respectively. First, unsaturated esters were prepared in the Wittig reaction and then transformed to 5-(bromomethylene)furan-2(5*H*)-ones in three different methods; A, B or C. The methods A and B comprised three steps whereas C comprised two. In method A, the esters were cleaved by trifluoroacetic acid in dichloromethane and then brominated to form α,β-dibromo acid. Thereafter the acid was debromodecarboxylated with triethylamine in dimethylformamide. Method A and B were similar, except that in B, the two first steps were switched. Method A gave somewhat better yield than B, but had a much longer reaction time. In method C, the unsaturated esters were cleaved to acids and then bromodecarboxylated by using bis(2,4,6-trimethylpyridine)bromine(I)hexafluorophosphate in dichloromethane. This reaction gave a low to moderate yield, but the reaction was rather stereo-specific. The 3-(bromomethylene)isobenzofuran-1(3*H*)-ones were formed using phtalic anhydrides as starting material in the methods described (Paper III). The furanones will later on be referred to by the names given to them in the studies; F101, F201-208, and F301-302.

Effect of furanones on biofilm formation

Having established the methods to synthesise furanones, the next aim was to assess their effect on biofilm formation.

F202 was investigated for ability to interfere with biofilm formation by *S. anginosus*, *S. intermedius*, and *S. mutans*, as well as by the isogenic AI-2 defective mutants of *S. anginosus* and *S. intermedius*. Biofilms were allowed to form on polyethylene surfaces with or without exposure to F202, which was either added to the growth medium or physically adsorbed to the surfaces.

F202 added to the growth medium at the concentrations 0.6 or 6.0 μM decreased biofilm formation in all three streptococci in a concentration dependent manner (Paper II).

Furanone coated wells were prepared by dissolving F202 to 60 mM in ethanol, and adding one millilitre of the dissolved furanone to wells of a polystyrene microtitre plate. The ethanol was left to evaporate in room temperature, leaving the furanone physically adsorbed to the surface of the wells. When surface adsorbed, F202 decreased biofilm formation by the streptococci to a higher degree than when added to the medium. Biofilm formation by *S. intermedius* and *S. mutans* on F202 adsorbed surfaces showed a substantial reduction, up to 76% compared to the control. This was confirmed by scanning electron microscopy (Paper II).

In a further biofilm assay where *S. epidermidis* was the biofilm forming microorganism, F302 was compared to the original F202. Biofilm was allowed to form on furanone adsorbed surfaces. Both F202 and F302 decreased the biofilm mass effectively, F202 being the most effective (Paper III).

Four different furanones F201-F202, F206 and F302, were further assessed for effect on *S. epidermidis* biofilm formation. This was done to identify the most promising furanones for the prevention of microbial biofilm infections. All four furanones tested gave significantly decreased biofilm mass. F202 however, decreased biofilm significantly more than the other three and was therefore included in further studies. F206, which contained 2 bromo substituents and therefore seemed structurally interesting, was also subjected to further studies (Paper IV).

Mechanism of action of furanones

The ability of the microorganisms to communicate via AI-2, and the possible interference with this communication by furanones, was assessed in a bioluminescence assay. In this assay,

induction of bioluminescence in the AI-2 reporter *V. harveyi* BB170, was used as a sensor of microbial communication via AI-2.

AI-2 production in *S. anginosus*, *S. intermedius*, *S. mutans*, *S. epidermidis,* and *V. harveyi* BB152, was confirmed by exposing their culture supernatants to the AI-2 biosensor. All streptococci (Paper II) and *S. epidermidis* (Paper IV) induced bioluminescence.

At the concentrations 0.6 and 6.0 μM, F206 decreased induction of bioluminescence by all streptococcal supernatants in a concentration dependent manner, with 6.0 μM almost completely inhibiting the AI-2 mediated induction of bioluminescence (Paper II).

The synthesised 5-(bromomethylene)furan-2(5*H*)-ones and 3-(bromomethylene) isobenzofuran-1(3*H*)-ones were also tested for interference of communication at 6.0 μM, with F202 included for comparison. One of the new furanones, F206, decreased AI-2 mediated induction of bioluminescence to a higher extent than F202 (Paper III).

Finally, 11 furanones (including the ones already tested in Paper II and Paper III) were tested in the same bioluminescence assay at 6.0 μM to find the most effective inhibitor of AI-2 mediated induction of bioluminescence. Supernatants collected from *V. harveyi* BB152 were exposed to the AI-2 biosensor *V. harveyi* BB170. Four of the furanones markedly decreased AI-2 mediated induction of bioluminescence (Paper IV).

The F202 concentrations used had no effect on *V. harveyi*, *S. anginosus*, *S. intermedius*, or *S. mutans* planktonic growth (biofilm combined with planktonic cells), or total growth of *S. anginosus*, *S. intermedius*, or *S. mutans* (Paper II).

The total growth was unaffected for all furanones used in the *S. epidermidis* biofilm assay (Paper III and Paper IV).

Minimum inhibitory concentration (MIC) for two selected furanones, F202 and F206, was approximately 16 and 26 times higher, respectively, than the concentrations necessary to inhibit biofilm formation (Paper IV).

When added to the growth medium, furanones had no effect on biofilm formation by the communication defective *S. anginosus luxS* mutant or the *S. intermedius luxS* mutant. The communication defective mutants were also allowed to form biofilm on furanone-coated surfaces. The biofilm formation by the *S. anginosus luxS* mutant was unaffected ($p>0.05$), while biofilm formed by the *S. intermedius luxS* mutant was slightly lower when formed on a furanone adsorbed surface (Paper II).

To further investigate whether the effect was related to interference with microbial communication, the AI-2 precursor (S)-4,5-dihydroxy-2,3-pentanedione (DPD) was added together with the furanones F202 and F206. The effect of the furanones was completely

abolished at the concentration 0.5 nM DPD, whereas DPD concentrations above and below 0.5 nM attenuated the effect (Paper IV).

Irritability and genotoxic assessments

F202 and F206 were tested for irritability in the HET-CAM assay. Both furanones were found to be harmless at the concentrations used to prevent biofilm formation. First at 6000 μM, the first signs of any irritative effect were seen. For F202, initial signs of coagulation were observed, and for F206 a small bleeding as well as initial signs of coagulation were observed (Paper IV).

Finally, possible genotoxic effects of F202 and F206 on global gene expression were investigated in mice. There were no clinical signs of adverse effects and no significant alterations in gene expression in the mice after ingestion of furanone for 21 days (Paper IV).

General discussion

Treatment of infections is today mainly based on antimicrobials. This may, however, in many cases not be adequate, since antimicrobials generally are developed and tested against planktonic microorganisms, disregarding the nature of microorganisms organised in a biofilm. The rapid spread of antimicrobial resistance seen today, is another challenge.

The discovery that microorganisms communicate with each other to coordinately regulate genes opens new possibilities to fight microbial infections. By targeting microbial communication systems, we may decrease biofilm formation and thereby prevent diseases caused by biofilms. It may thus be easier to subsequently treat infections with relevant antimicrobials as the targeted microorganisms will be in planktonic phase.

During the work of this study, we have investigated whether furanones would be potential agents for preventing biofilm formation in Gram-positive microorganisms (Paper II, Paper III and Paper IV).

Furanone synthesis

To consider furanones as potential agents for preventing biofilm associated infections, there was a need to find easy and inexpensive ways to synthesise furanones. Most studies on furanone interference with microbial communication have been based on natural furanones, whereas only few are based on synthetic furanones (Hentzer *et al.*, 2002; Manefield *et al.*, 2002; Ren *et al.*, 2004; Ren *et al.*, 2004; Wu *et al.*, 2004).

First, we tried to synthesise a natural furanone that earlier had been tested for interference with microbial communication in *Pseudomonas aeruginosa* (Wu *et al.*, 2004). This compound was named F202 in the present study (see Fig. 1 for structure). We designed a new and general way to synthesise different compounds, including F202 (Paper I). The scheme was simpler, and with a higher yield than by previously published schemes (Manny *et al.*, 1997). Sorg and co-workers however published a new scheme in 2005. This scheme gave similar yield, but was less general (Sorg *et al.*, 2005).

Since *Delisea pulchra* is known to synthesise furanones of various structures, it was further interesting to see whether we could increase the efficacy by altering the structure of our furanones. Based on the synthesis pathway for F202, we were able to synthesise ten more furanones (F101, F201, F203-F208, and F301-F302) with the original main structure but with different side chains. We also varied the position of the halogen (F205, F207 and F301), as

well as exchanged halogen (F201). We also included two bromines in one furanone (F206) (Paper III). (See Fig. 1 for structures.)

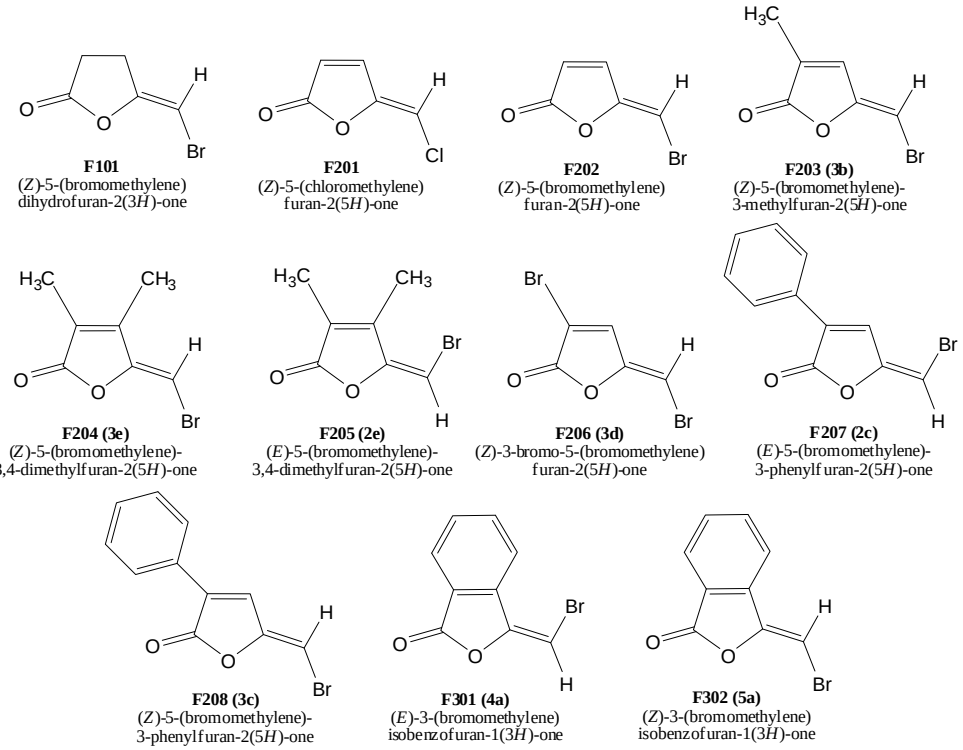

Figure 1: Chemical structures of the furanones included in this study.

Effect of furanones on biofilm formation

The discovery that microorganisms prefer a biofilm mode of growth rather than a free floating life style, and that they regulate their behaviour through communication, has changed the way we look at microorganisms. So far, we are only in the early beginning of using this knowledge in the prevention and treatment of biofilm associated diseases.

In previous studies of furanone efficacy, the furanones have been added to the growth medium (Ren *et al.*, 2001; Ren *et al.*, 2004) or to already existing biofilms (Hentzer *et al.*, 2002; Hentzer *et al.*, 2003; Wu *et al.*, 2004). We started our study by adding the compound F202 to the growth medium to investigate a possible inhibitory effect on biofilm formation. Since biofilm related infections often are connected to implants, we investigated whether a furanone covered surface would prevent biofilm formation, to the same degree as when adding the furanone to the growth medium (Paper II). This has been done earlier with other

furanone compounds, both *in vitro* and in mouse models (Hume *et al.*, 2004; Baveja *et al.*, 2004 a; Baveja *et al.*, 2004 b). We used a simple coating procedure where the furanones were dissolved in ethanol, which was then let to evaporate, leaving furanone physically adsorbed to the surface. For all streptococcal strains tested, this showed to be more efficacious than when adding furanone to the growth medium. The adsorbed furanone also effectively decreased *S. epidermidis* biofilm formation (Paper III, Paper IV). It is possible that part of the adsorbed furanone may dissolve and leak into the growth medium. The maximum possible furanone concentration by complete dissolution is 60 μM and would be antimicrobial. No antimicrobial effect was seen, however, since total growth, including both biofilm and planktonic cells, was unaffected. With the present coating procedure we cannot control how evenly the surface is coated or whether the entire surface is actually covered. There is therefore a future need to monitor the stability and to develop stable furanone films with even distribution of the active molecules.

The efficacy of the furanones differed between the different structures; F202 decreased biofilm formation more than the other furanones tested (Paper IV). This was the case for all microbial strains included. Difference in efficiency between different structures has also been seen in studies of *E. coli* biofilm formation (Han *et al.*, 2008) as well as in *Salmonella enterica* (Janssens *et al.*, 2008).

Han and co-workers concluded that the most important structural element of the furanone for biofilm inhibition in *E. coli* is the bromine atom in the Z-position of the furanone ring. This is in accordance with our observations for streptococcal species (data not published), as well as the results of the bioluminescence assay comparing interference by F301 and 302 (Paper III, Paper IV). In *Salmonella*, the furanones lacking an alkyl chain were the most effective inhibitors of biofilm formation, but at the same time they also delayed planktonic growth at low concentrations (Janssens *et al.*, 2008). We also found the furanones lacking an alkyl chain to be most effective in decreasing biofilm formation. However, we observed no effect on growth at the concentrations used in our biofilm assays. Furthermore, the MICs established for the furanones F202 and F206 were more than twice that found for *Salmonella* (Janssens *et al.*, 2008). Janssens and co-workers results also indicate that the biofilm inhibitory effect of the furanone decreased as the length of the alkyl side chain increased. These indications of structure dependent efficacy underline the need for more detailed structural screening studies. So far it seems crucial that a halogen is included in the structure and the position might be important. The length of the alkyl side chains also seems to be relevant (Paper IV).

Mechanism of action of furanones

For possible clinical use of furanones, it is crucial to understand the mechanisms of action. This was examined by the following approaches, 1) investigating a possible antimicrobial effect, 2) examining interference with microbial communication, specifically AI-2 communication, 3) assessing the inhibitory effect of furanone on AI-2 communication defective mutants, and finally 4) supplementation of synthetic communication signal, DPD, for competition with furanone.

Antimicrobial effect

All furanone concentrations used in the bioluminescence and biofilm assays were well below concentrations that had any effect on microbial growth (Paper II, Paper III, Paper IV). Biofilm formation by *E. coli* (Ren *et al.*, 2001) and *Salmonella* (Janssens *et al.*, 2008) was also inhibited by furanones at concentrations without an effect on growth. For the two compounds, F202 and F206, that showed the most promising results in the biofilm assay, we determined MIC to be 1000 and 1600 μM respectively. For both compounds, the maximum concentration that the microorganisms could possibly be exposed to through dissolution of the surface adsorbed furanone was 60 μM, well below the established MICs (Paper IV). Although 60 μM would restrain growth in some microorganisms, in none of our biofilm assays was the total growth affected, indicating that not all furanone was dissolved during the time of the assay. By this same coating method, Baveja and co-workers found that between 10-100 % of furanone remained on the surface after 24 hours incubation, depending on the surface material (Baveja *et al.*, 2004 a). The anti-biofilm effect found in the present study could therefore most probably not be ascribed to an antimicrobial effect. Since growth was unaffected, the furanones will most likely not exert any selective pressure that may lead to resistance development.

Microbial communication

The *luxS* gene seems to be important for communication in a wide variety of microorganisms, and homologues have been found in all species used in this study (Merritt *et al.*, 2003; Yoshida *et al.*, 2005; Petersen *et al.*, 2006; Xu *et al.*, 2006; Ahmed *et al.*, 2008). Through the bioluminescence assay, we reconfirmed that AI-2 is produced by the streptococcal strains (Blehert *et al.*, 2003; Merritt *et al.*, 2003; Wen and Burne, 2004; Yoshida *et al.*, 2005; Petersen *et al.*, 2006) as well as *S. epidermidis* (Xu *et al.*, 2006), indicating that these species communicate via AI-2 signalling (Paper II).

The synthesised furanones clearly decreased induction of bioluminescence in *V. harveyi* BB170. Since this could not be ascribed to an antimicrobial effect, we assume that the furanones were able to interfere with microbial communication (Paper II, Paper III, Paper IV). An indication of AI-2-specific interference was that the furanones decreased bioluminescence induced in *V. harveyi* BB170, sensing only AI-2. Our results thus support previous findings of furanones decreasing bioluminescence mediated by AI-2 (Ren *et al.*, 2004). The bioluminescence assay is an indirect method of detecting AI-2 signal molecules. Bioluminescence results should therefore be interpreted with caution (Turovskiy and Chikindas, 2006). Thus the exact mechanism of the anti-biofilm effect of the furanones seen in the studied microorganisms remains to be elucidated. Recent studies indicate that the quorum sensing regulator of *V. harveyi*, LuxR, (Waters and Bassler, 2006; Tu and Bassler, 2007), is altered by furanone, making it unable to bind to the promoter of quorum sensing regulated genes (Defoirdt *et al.*, 2007). By searching the genome of *S. epidermidis* we found two open reading frames encoding putative response-regulators belonging to the LuxR family. The function of these reading fames are however presently unknown, as well as their possible involvement in AI-2 communication.

Wild type vs. *luxS* mutant

In the streptococcal biofilm assay, we compared biofilm formation by the wild types and their corresponding *luxS* mutants (Paper II). We confirmed that the mutants formed less biofilm than their wild type counterpart (Petersen *et al.*, 2006). When adding furanone to the growth medium, biofilm formation by the communication deficient *luxS* mutants of both *S. anginosus* and *S. intermedius* remained unaffected. The wild types that were able to communicate via AI-2 produced less biofilm in presence of furanone, supporting the effect on AI-2 communication (Paper II). This provided further circumstantial evidence of a furanone effect through communication interference.

When biofilm was grown on furanone adsorbed surfaces, the *S. anginosus luxS* mutant was still unaffected. Biofilm formation by the *S. intermedius luxS* mutant was decreased to some degree but less than by its wild type. Assuming that furanone affects biofilm formation through AI-2 communication interference, the decreased biofilm formation is difficult to explain. Biofilm formation involves several steps, however. The *S. intermedius* mutant could possibly escape the effect of furanone by alternative pathways, since streptococci may use several other pathways to regulate biofilm formation, including peptide communication systems (Petersen *et al.*, 2004).

DPD

Even though AI-2 communication is found in a vast number of species, the AI-2 molecule seems to be diverse in different species. Most likely, the molecules are derived from the same precursor, (*S*)-4,5- dihydroxy-2,3-pentanedione (DPD) (Miller *et al.*, 2004). DPD is highly reactive and rearranges spontaneously into a range of similar structures, collectively called AI-2 (Vendeville *et al.*, 2005; De Keersmaecker *et al.*, 2006). The AI-2 structures of *V. harveyi* and *Salmonella typhimurium* have been identified (Fig. 2), whereas the AI-2 in staphylococci and streptococci are still unknown.

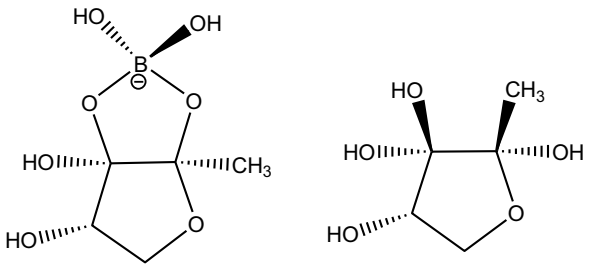

Figure 2: Chemical structures of A1-2 from *V. harveyi* (left) (Chen *et al.*, 2002) and *S. typhimurium* (right) (Miller *et al.*, 2004).

DPD is commercially available and by addition of DPD the effects of the two furanones on biofilm formation were eliminated. This was a strong indication that the furanones affect biofilm formation through interference with AI-2 microbial communication. Consistent with DPD being a quorum sensing signal, we found that the concentration needed to abolish the inhibitory effect of furanone was very low. There was also a narrow range of concentrations where DPD abolished the effect of the furanones (Paper IV). This is in line with previous studies, where it has been observed that a narrow range of concentrations of DPD induced a quorum-sensing response (Rickard *et al.*, 2006; Ahmed *et al.*, 2008).

Xu and co-workers reported in a previous study that the *luxS* mutant of *S. epidermidis* formed more biofilm than its wild type (Xu *et al.*, 2006). This result opposes a furanone effect through inhibition of AI-2 communication and is in contrast with our results, showing that DPD competes with furanone and restores the ability of *S. epidermidis* to form biofilm. These contradictory results are difficult to explain, but could be related to factors such as biofilm growth conditions, e.g. growth medium, culture well materials, and quantification

methods. More recently, the same authors showed that externally added AI-2 almost completely restored the gene expression pattern of the wild type in *S. epidermidis luxS* mutant. They also found that AI-2 regulated the expression of phenol-soluble modulins, which seem to have a function in biofilm development (Li *et al.*, 2008). This would support our assumption that furanones interfere with AI-2 mediated communication in *S. epidermidis* as well, although the role of AI-2 in *S. epidermidis* biofilm formation is still unresolved.

It was previously assumed that furanones functioned as competitive inhibitors of AI-2. The structures of the known AI-2 molecules carry some similarity to the furanone compounds used in our studies (Fig. 1, Fig. 2). The present view of the furanone effect in *V. harveyi* is, however, that the gene regulator LuxR is prevented from binding to its promoter sequence (Defoirdt *et al.*, 2007). To elucidate how the furanones function on a molecular-genetic level in Gram-positive microorganisms, such as the strains used in this study, requires further research.

Furanones irritability and genotoxicity

The next goal, after having identified two furanones that effectively decreased biofilm formation without an antimicrobial effect (F202 and F206), was to further assess their suitability for future clinical use. There are several studies on the potential of furanones to decrease biofilm formation (Ren *et al.*, 2001; Hentzer *et al.*, 2002; Ren *et al.*, 2002; Hume *et al.*, 2004; Baveja *et al.*, 2004 a; Baveja *et al.*, 2004 b; Christensen *et al.*, 2007; Janssens *et al.*, 2008; Kim *et al.*, 2008). There are, however, only few studies on the suitability for clinical use. These concern eukaryotic cell viability and toxicity, (Hume *et al.*, 2004; Rasch *et al.*, 2004; Baveja *et al.*, 2004 a; Baveja *et al.*, 2004 b), whereas reports on irritative or genotoxic effects seems to be lacking. To consider whether furanones would be of clinical use against biofilm related diseases, there is a need for more thorough studies on possible adverse effects.

To get a first impression on the suitability for clinical use, we tested the two compounds F202 and F206 for a possible irritative effect (Paper IV). To predict any irritative effect on the sensitive tissue surrounding a medical device, we used the HET-CAM method. This method, where the blood vessels of the CAM are exposed to the chemical, is comparable to the accepted and commonly used Draize eye irritation test (Balls *et al.*, 1995). A score was calculated, taking all reactions of the CAM into consideration. From this score, an irritative potential was assessed (Kalweit *et al.*, 1990). The first signs of irritative effects were not observed until a concentration of 6000 µM was used. This concentration was more than 100 times higher than that necessary to affect biofilm formation.

A first attempt to assess possible genotoxic effects of furanones was made by using a mouse model (Paper IV). Genotoxicity was assessed as toxic reactions in mice using a membrane array comprising genes associated with toxic responses, followed by a study of the effect of the furanones on global gene expression using microarrays. None of the genes associated with toxic responses were significantly altered in expression by the furanones at concentrations decreasing biofilm formation. This was taken as evidence of no toxic responses to ingestion of the furanones by mice after 3 or 21 days. There were furthermore no signs of significant alterations in the expression of the 30 000 genes of the mouse genome by the furanones. This indicates that furanones could be interesting alternatives or adjuncts to the antimicrobials commonly used today. There is, however, need for further studies on the toxicology, including long-term studies and studies on offspring, as well as studies of delivery routes and vehicles.

Concluding remarks

This thesis addresses a novel approach in the prevention of microbial biofilm related infections through interference with microbial quorum sensing communication by synthetic furanones. The rationale rests on the knowledge of biofilm formation as a well-regulated process. To form complex biofilm communities, the microorganisms coordinate their behaviour through quorum sensing communication. The resilient nature of microbial biofilms accounts for their causative role in persistent implant infections. Traditional treatment of these infections are generally targeted against planktonic cells, and are therefore often less suitable for treating biofilm related infections. Synthetic furanones that target microbial communication, such as AI-2 mediated communication, may decrease biofilm formation and virulence, without exerting a selective pressure and without adverse effects. Interference with microbial communication thus may represent a novel and promising strategy to control microbial biofilm infections.

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I

II

III

Synthesis of 5-(bromomethylene)furan-2(5*H*)-ones and 3-(bromomethylene)isobenzofuran-1(3*H*)-ones as inhibitors of microbial quorum sensing

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(*E*)- and (*Z*)-5-(Bromomethylene)furan-2(5*H*)-ones and (*E*)- and (*Z*)-3-(bromomethylene)isobenzofuran-1(3*H*)-ones have been prepared starting from commercially available maleic anhydrides and phthalic anhydrides, respectively. A debrominative decarboxylation or a bromodecarboxylation reaction is a key step in the synthesis. The furanones were investigated for their ability to interfere with microbial communication and biofilm formation by *Staphylococcus epidermidis*.

Introduction

Many microorganisms communicate *via* chemical signal molecules such as autoinducer-1 (AI-1) and autoinducer-2 (AI-2) to control gene expression in response to population density.[1] This phenomenon, called quorum sensing (QS), regulates various virulence factors, for instance proteolytic activity, carbohydrate metabolism and biofilm formation.[2,3] Thus interference with QS, in principle, would reduce the pathogenic potential of a bacterium. Since this occurs without exerting a selective pressure on microbial viability, resistance development is not likely.

Most microorganisms in nature prefer a biofilm mode of growth[4] and biofilm formation is regulated by QS in a number of microorganisms.[5,6] *Staphylococcus epidermidis* is a Gram-positive biofilm forming commensal bacterium on human skin and mucous membranes, as well as a major nosocomial pathogen associated with medical implant infections.[7] The virulence is related to *S. epidermidis*'s ability to form biofilm on implanted devices. There are presently few effective means to prevent medical implant infections,[8] mainly due to increased resistance of biofilm microorganisms to both antimicrobials and the human immune system, being up to 10–1000 times more tolerant to antimicrobials than their planktonic counterparts.[9] Thus antimicrobial treatment of implant infections often fails, necessitating removal of the implanted device. Control of biofilm formation by for instance *S. epidermidis* represents a novel, non-antimicrobial approach of implant infection prevention. Thus identification of compounds that inhibit QS has become an area of intense research.[2] The macro algae *Delisea pulchra* is known to prevent microbial colonisation of its surface by producing brominated furanones.[10] Such furanones are thought to interfere with microbial communication, mainly in Gram-negative microorganisms.

The aim of this study was to synthesize 5-(bromomethylene)furan-2(5*H*)-ones (**2** and **3**, Scheme 1) and 3-(bromomethylene)isobenzofuran-1(3*H*)-ones (**4** and **5**, Scheme 2) and to assess their ability to interfere with microbial communication and biofilm formation by *S. epidermidis*.

5-(Bromomethylene)furan-2(5*H*)-ones have previously been prepared from levulinic acid[11] or its derivatives,[12] propenoates[13] and allenic esters.[14] 3-(Bromomethylene) isobenzofuran-1(3*H*)-ones have been prepared from 3-alkylidene isobenzofuran-1(3*H*)-ones[15] and by dehydration of bromo keto acids.[16]

5-(Alkylidene)furan-2(5*H*)-ones, without the bromo substituent in the alkylidene group, have been synthesized from maleic anhydrides in a Wittig reaction using stabilized phosphorus ylides.[17] We wanted to use this method in our synthesis since a number of maleic and phthalic anhydrides are commercially available. The necessity to use stabilized phosphorus ylides in the Wittig reaction indicates a bromodecarboxylation step of an α,β-unsaturated acid in order to prepare the 5-bromomethylene substituent. A number of methods have been used in the bromodecarboxylation of α,β-unsaturated acids having a β-aryl substituent.[18] Without such a substituent the methods are much more limited.[19]

Results and discussion

Synthesis of furanones

The unsaturated esters **1** (Scheme 1) were prepared in good yields from commercially available maleic anhydrides in the Wittig reaction and the esters were transformed into the 5-(bromomethylene)furan-2(5*H*)-ones (**2** and **3**) in three ways (Method A, B and C, Table 1). Methods A and B have three steps, while Method C has two. All methods are one-pot procedures—with only solvent evaporation between the steps.

In Method A the *tert*-butyl ester of **1** is first cleaved by TFA in dichloromethane and then brominated before the resulting

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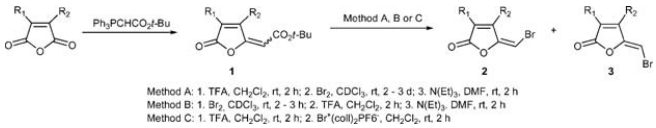
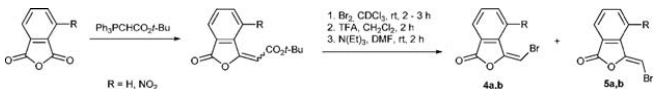


Scheme 1



Scheme 2

α,β-dibromo acid is debromodecarboxylated with triethylamine in DMF. Method B is similar to Method A except that the two first steps are switched. The yields in Method A are somewhat better than the yields in Method B, but the reaction times in Method A are much longer than in Method B. The bromination of the unsaturated acids takes 3–4 days with a large excess of bromine, while the bromination of the unsaturated esters is over in a couple of hours with a slight excess of bromine. The product distribution of the *E*- and *Z*-isomer of the 5-(bromomethylene)furan-2(5*H*)-one (**2** and **3**) is almost the same in these two methods (Table 1). The *Z*-isomer is always the major isomer except in one case (R_1 and R_2 = Me). The identity of the *E*- and *Z*-isomers were determined by NOE experiments and/or by ^{1}H NMR chemical shift of the methine proton and any ring protons.[20]

In Method C the unsaturated esters are cleaved to the acids and then bromodecarboxylated using bis(2,4,6-trimethylpyridine)bromine(I) hexafluorophosphate in dichloromethane. The yield in this reaction is low to moderate, 10–58% over two steps (Table 1). The reaction shows some degree of stereospecificity since treatment of the pure *E*-isomer of the corresponding acid of **1** (R_1 = R_2 = Me) gives mainly the *E*-isomer (*E* : *Z* ratio 71 : 29) of the bromodecarboxylated product. The pure *Z*-isomer gives only the *Z*-isomer.

The product ratios in Table 1 are probably not equal to the thermodynamic ratios of the products, since treatment of either pure **2c** or pure **3c** with iodine in $CDCl_3$ gives the same mixture of **2c** and **3c** (29 : 71) which is significantly different from the ratio in Table 1 (12 : 88)

Phthalic anhydrides can also be used as starting material in the synthesis discussed above. Thus the 3-(bromomethylene)-isobenzofuran-1(3*H*)-ones **4a** and **5a** (R = H, Scheme 2) were formed as a 1 : 2 mixture in 53% yield while the nitro compounds **4b** and **5b** (R = NO_2, Scheme 2) were formed as a 1 : 1 mixture in 27% yield. We could separate the *E*- and *Z*-isomers **4a** and **5a** by flash chromatography but not the nitro compounds **4b** and **5b**.

Bromination of the unsaturated ester **1** (Method B, step 1) or the corresponding unsaturated acid (Method A, step 2) is completely regioselective: only the exocyclic carbon–carbon double bond is brominated at room temperature even if an excess of bromine is used.

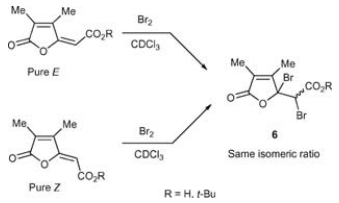
Bromination of ordinary alkenes is stereospecific.[22] The bromination of the unsaturated ester **1** (R_1 = R_2 = Me) or the corresponding unsaturated acid is, however, not stereospecific because the pure *E*-isomer and the pure *Z*-isomer of **1** give the same mixture of dibrominated ester or acid (Scheme 3).

The isomerization occurs only in the bromination step, since no isomerization was observed when a pure dibromide

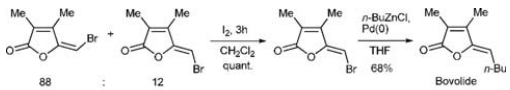
Table 1 Yields and product distribution in the synthesis of **2** and **3**

R_1	R_2	*E* : *Z* Starting material **1**	Method[a]	Product	Yield[b] **2**	Yield[b] **3**	Total yield[b]	*E* : *Z* Product **2** : **3**
H	H	100 : 0	A	**2a**/**3a**[c]	—	—	61	5 : 85
H	H	100 : 0	B	**2a**/**3a**[c]	2	44	46	4 : 96
H	H	100 : 0	C	**2a**/**3a**[c]	22	0	22	100 : 0
Me	H	100 : 0	A	**2b**/**3b**	10	77	87	11 : 89
Me	H	100 : 0	B	**2b**/**3b**	7	46	53	13 : 87
Me	H	100 : 0	C	**2b**/**3b**	32	6	38	84 : 16
Ph	H	100 : 0	A	**2c**/**3c**	7	53	60	12 : 88
Ph	H	100 : 0	B	**2c**/**3c**	6	45	51	12 : 88
Ph	H	100 : 0	C	**2c**/**3c**	54	4	58	93 : 7
Br	H	100 : 0	A	**2d**/**3d**	6	50	56	11 : 89
Br	H	100 : 0	B	**2d**/**3d**	1	48	49	2 : 98
Br	H	100 : 0	C	**2d**/**3d**	6	4	10	60 : 40
Me	Me	0 : 100	A	**2e**/**3e**	66	9	75	88 : 12
Me	Me	0 : 100	B	**2e**/**3e**	52	14	66	79 : 21
Me	Me	0 : 100	C	**2e**/**3e**	—	—	—	0 : 100[d]
Me	Me	100 : 0	C	**2e**/**3e**	34	14	48	71 : 29

[a] Method A: 1. TFA, CH_2Cl_2, rt, 2 h; 2. Br_2, $CDCl_3$, rt, 2–3 d; 3. $N(Et)_3$, DMF, rt, 2 h; Method B: 1. Br_2, $CDCl_3$, rt, 2–3 h; 2. TFA, CH_2Cl_2, 2 h; 3. $N(Et)_3$, DMF, rt, 2 h; Method C: 1. TFA, CH_2Cl_2, rt, 2 h; 2. $Br^+(coll)_2PF_6^-$, CH_2Cl_2, rt, 2 h. [b] Isolated yield. [c] Ref. 21. [d] From the ^{1}H NMR of the crude product.



Scheme 3



Scheme 4

(**6**, R = *t*-Bu) was carried through to the end product. The 5-bromomethylenefuran-2(5*H*)-ones will, however, isomerize if the compounds are kept in a polar solvent like $CDCl_3$ for a couple of weeks. This tendency to isomerize can be utilized in a synthesis of the natural product bovolide[23] (**7**, Scheme 4). A mixture of **2e** and **3e** was isomerized to pure **3e** with iodine in CH_2Cl_2 and coupled with butylzinc chloride in THF at room temperature in a Pd(0)-catalyzed reaction.

The brominated furanone **3d** (Table 1) has proven to be a valuable intermediate in the synthesis of another γ-alkylidene butenolide: lissoclinolide.[24]

Interference with microbial communication and biofilm formation

All synthesized furanones (**2e**, **3b–e**, **4a**, **5a**) and the reference **F202**[21] (Fig. 1) at 6.0 μM reduced bioluminescence in *V. harveyi* BB170 significantly ($P < 0.01$ compared to control without furanone), with **5a** being slightly more effective than the **F202** reference (Fig. 2).

V. harveyi BB170 lacks the receptor for AI-1 and thus responds only to intermicrobial communication *via* the AI-2 QS molecule. We assume therefore that the tested furanones interfered with AI-2 QS communication. This is in line with previous data in both Gram-negative[25] and Gram-positive microorganisms.[26] More recently, furanones were found to structurally alter LuxR in *V. harveyi* thus preventing binding to promoter sequences.[27] Further studies are needed to verify the mechanism of action against *S. epidermidis*.

The two most effective bioluminescence reducers (**5a** and **F202**) were subsequently tested for effect on biofilm formation by *S. epidermidis*. *S. epidermidis* carries the AI-2 synthase gene, although its role in biofilm formation is not clearly defined. The biofilm assay clearly showed the biofilm inhibitory potential of both furanone **5a** and **F202** ($P < 0.01$ compared to control without furanone). Furanone **5a** reduced *S. epidermidis* biofilm by 57%, while the reference **F202** reduced biofilm formation by

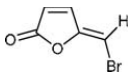


Fig. 1 Structure of compound **F202**, (*Z*)-5-(bromomethylene)furan-2(5*H*)-one.[21]

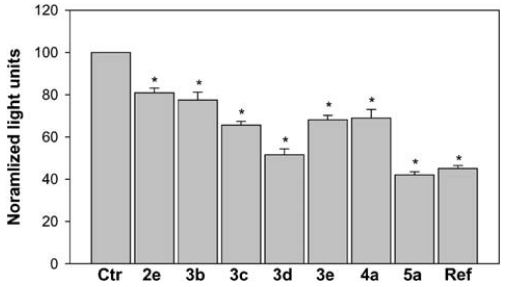


Fig. 2 Bioluminescence response in the reporter strain *V. harveyi* BB 170 induced by *V. harveyi* BB152 supernatant and repressed by 6.0 μM of furanones **2e**, **3b–e**, **4a**, **5a** or the reference **F202**. The results are mean values and standard errors from three independent experiments with three parallels. * Significantly different from control (ctr) without furanone ($P < 0.01$).

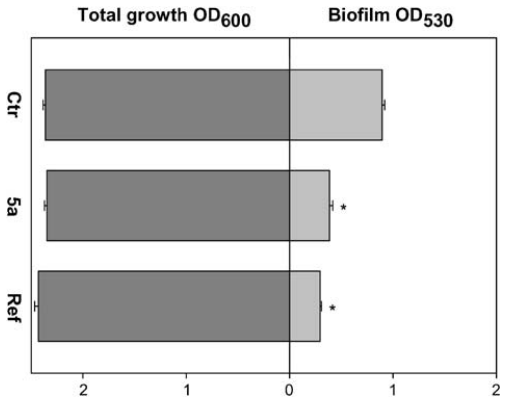


Fig. 3 24 h Biofilm formation by *S. epidermidis* on discs coated with furanone **5a** and the reference **F202**. The results are mean values and standard errors from three independent experiments done in triplicate.* Significantly different from control (ctr) without furanone ($P < 0.01$).

68%. Notably, this reduction could not be ascribed to an antimicrobial effect since for both compounds, total growth was unaffected (Fig. 3). These findings are interesting in view of the increased use of implanted medical devices and the concomitant implant infections. We suggest that synthetic compounds like **5a** and **F202**, immobilised on implant surfaces, may prevent implant infection. **F202** was more effective than **5a** in reducing biofilm formation by *S. epidermidis*. **F202** has previously been reported to interfere with colonization by the Gram-negative *Pseudomonas aeruginosa* in lungs of experimentally infected mice.[28] Thus **F202** appear to be able to interfere with both AI-1 and AI-2 communication. The present and other studies confirm the possibility of interfering with microbial virulence without inhibiting microbial growth.

Conclusion

We have shown that 5-(bromomethylene)furan-2(5*H*)-ones and 3-(bromomethylene)isobenzofuran-1(3*H*)-ones can be

easily synthesized in 3 or 4 steps from commercially available maleic anhydrides and phthalic anhydrides, respectively. This study also shows that furanones may be potential inhibitors of microbial communication. The most effective furanones, **5a** and **F202**, also reduced biofilm formation by *S. epidermidis* without affecting the growth. The bioluminescence assay indicates that the efficacy may be structurally dependent. We see a potential for furanones and isobenzofuranones in preventing implant infections.

Experimental

The ^{1}H NMR and the ^{13}C NMR spectra were recorded on Bruker Avance DPX instruments. Mass spectra, under electron impact conditions, were recorded at 70 eV ionizing energy on a Fision ProSpec instrument.

Preparation of 5-(bromomethylene)furan-2(5*H*)-ones and 3-(bromomethylene)isobenzofuran-2(3*H*)-ones

Method A. The α,β-unsaturated ester (1.0 mmol) was dissolved in a mixture of CH_2Cl_2 (1 mL) and TFA (1 mL). The reaction mixture was stirred at room temperature for 2 h, evaporated and redissolved in a mixture of $CDCl_3$ (2 mL) and TFA (0.1 mL). Bromine (2 mL, 2 M in CCl_4) was added and the mixture was stirred at room temperature until ^{1}H NMR showed that all starting material had been consumed (2–3 d). The solvents were evaporated off and the residue was dissolved in DMF (2 mL). Triethylamine (0.15 mL, 1.08 mmol) was added at 0 °C and the mixture stirred at room temperature for 45 min before water was added. The product was extracted into Et_2O, washed with brine (3 × 10 mL), dried ($MgSO_4$) and evaporated. The *E*- and *Z*-isomers were separated by flash chromatography on silica gel.

Method B. Bromine (0.55 mL, 1.10 mmol, 2 M in CCl_4) was added to a solution of the α,β-unsaturated ester (1.0 mmol) in CH_2Cl_2 (2 mL). The mixture was stirred at room temperature for 2 h before the solvent was evaporated and the residue dissolved in a mixture of CH_2Cl_2 (1 mL) and TFA (1 mL). The mixture was stirred at room temperature for 2 h before the solvent was evaporated off. The residue was dissolved in DMF (2 mL) and triethylamine (0.15 mL, 1.08 mmol) was added at 0 °C. The mixture was stirred at room temperature for 45 min before water was added and the product was extracted into Et_2O, washed with brine (3 × 10 mL), dried ($MgSO_4$) and evaporated. The *E*- and *Z*-isomers were separated by flash chromatography on silica gel.

Method C. The α,β-unsaturated ester (1.0 mmol) was dissolved in a mixture of CH_2Cl_2 (1 mL) and TFA (1 mL). The reaction mixture was stirred at room temperature for 2 h, evaporated and redissolved in CH_2Cl_2 (4 mL). Bis(2,4,6-trimethylpyridine)bromine(ı) hexafluorophosphate[29] (700 mg, 1.50 mmol) was added at 0 °C and the mixture was stirred at 0 °C for 15 min and at room temperature for 2 h before Et_2O was added. The Et_2O was washed with 1M HCl, saturated $NaHCO_3$ and brine before it was dried ($MgSO_4$) and evaporated. The *E*- and *Z*-isomers were separated by flash chromatography on silica gel.

(*E*)-5-(Bromomethylene)-3-methylfuran-2(5*H*)-one (2b). Eluent EtOAc–hexane 1 : 6 R_f 0.30; mp 52–56 °C; δ_H (300 MHz; $CDCl_3$) 2.01 (3H, Me), 6.33 (1H, s, CHBr), 7.37 (1H, m, H4); δ_C (75 MHz; $CDCl_3$) 11.03, 91.96, 133.42, 134.52, 150.93, 170.02; *m*/*z* (EI) 190 (M^+ + 2, 63%), 188 (M^+, 64), 125 (65), 122 (40), 120 (42), 97 (42), 68 (49), 39 (100); HRMS (EI) calcd. for $C_6H_5O_2Br$: 187.9472, found 187.9467.

(*Z*)-5-(Bromomethylene)-3-methylfuran-2(5*H*)-one (3b). Eluent EtOAc–hexane 1 : 6 R_f 0.13; mp 91–94 °C; δ_H (200 MHz; $CDCl_3$) 1.95 (3H, Me), 5.91 (1H, s, CHBr), 7.03 (1H, m, H4); δ_C (75 MHz; $CDCl_3$) 10.75, 89.57, 131.24, 135.81, 151.18, 169.52; *m*/*z* (EI): 190 (M^+ + 2, 99%), 188 (M^+, 100), 162 (16), 160 (16), 122 (55), 120 (57), 53 (76); HRMS (EI) calcd. for $C_6H_5O_2Br$: 187.9472, found 187.9469.

(*E*)-5-(Bromomethylene)-3-phenylfuran-2(*H*)-one (2c). Eluent EtOAc–hexane 1 : 3 R_f 0.48; δ_H (200 MHz; $CDCl_3$) 6.48 (1H, s, =CHBr), 7.41–7.47 (3H, m, Ph), 7.82 (1H, s, H4), 7.91–7.96 (2H, m, Ph); δ_C (75 MHz; $CDCl_3$) 93.89, 124.50, 128.78, 128.95, 130.43, 131.12 , 133.15, 150.75, 167.74; *m*/*z* (M^+) 252 (M^+ + 2, 59%), 250 (M^+, 60), 171 (100), 115 (74), 102 (88), 57 (20); HRMS (EI) calcd. for $C_{11}H_7O_2Br$: 249.9629, found 249.9626.

(*Z*)-5-(Bromomethylene)-3-phenylfuran-2(*H*)-one (3c). Eluent EtOAc–hexane 1 : 3 R_f 0.32; mp 123–125 °C; δ_H (200 MHz; $CDCl_3$) 6.10 (1H, s, =CHBr), 7.40–7.44 (3H, m, Ph), 7.47 (1H, s, H4), 7.85–7.90 (2H, m, Ph); δ_C (75 MHz; $CDCl_3$) 91.58, 127.15, 128.69, 128.91, 130.13, 131.30, 132.50, 151.03, 167.1; *m*/*z* (EI): 252 (M^+ + 2, 68%), 250 (M^+, 69), 172 (14), 171 (100), 116 (10), 115 (93), 102 (100), 76 (17); HRMS (EI) calcd. for $C_{11}H_7O_2Br$: 249.9629, found 249.9623.

(*E*)-3-Bromo-5-(bromomethylene)furan-2(5*H*)-one (2d). Eluent EtOAc–hexane 1 : 6 R_f 0.23; mp 52–55 °C; δ_H (200 MHz; $CDCl_3$) 6.53 (1H, s, =CHBr), 7.83 (1H, s, H4); *m*/*z* (EI) 256 (M^+ + 4, 49%), 254 (M^+ + 2, 100), 252 (M^+, 52), 228 (9), 226 (19), 224 (10), 175 (11), 173 (11), 149 (15), 147 15), 145 (15), 122 (18), 120 (19) 119 (17), 117 (17).

(*Z*)-3-Bromo-5-(bromomethylene)furan-2(5*H*)-one (3d).[24] Eluent EtOAc–hexane 1 : 6 R_f 0.15; mp 73–76 °C; δ_H (300 MHz; $CDCl_3$) 6.19 (1H, s, =CHBr), 7.49 (1H, s, H4); δ_C (75 MHz; $CDCl_3$) 93.27, 114.42, 139.42, 150.80, 164.01; *m*/*z* (EI) 256 (M^+ + 4, 49%), 254 (M^+ + 2, 100), 252 (M^+, 51), 228 (8), 226 (16), 224 (8), 145 (16), 147 (15), 53 (37). HRMS (EI) calcd. for $C_5H_2O_2Br_2$: 251.8421, found 251.8422.

(*E*)-5-(Bromomethylene)-3,4-dimethylfuran-2(5*H*)-one (2e). Eluent EtOAc–hexane 1 : 4 R_f 0.40; mp 48–50 °C; δ_H (200 MHz, $CDCl_3$) 1.87 (3H, m, 4-Me), 2.35 (3H, m, 3-Me), 6.38 (1H, s, =CHBr); δ_C (75 MHz, $CDCl_3$) 8.8, 13.7, 91.3, 129.0, 146.2, 150.8, 169.0; *m*/*z* (EI) 204 (M^+ + 2, 100%), 202 (M^+, 100), 191(9), 189(9), 139(10), 127 (17), 122 (26), 120 (26); HRMS (M^+) calcd. for $C_7H_7O_2Br$: 201.9629, found 201.9631.

(*Z*)-5-(Bromomethylene)-3,4-dimethylfuran-2(5*H*)-one (3e). Eluent EtOAc–hexane 1 : 4 R_f 0.26; mp 113–116 °C; δ_H (200 MHz; $CDCl_3$) 1.92 (3H, m, 4-Me), 2.10 (3H, m, 3-Me), 5.98 (1H, s, =CHBr); δ_C (75 MHz; $CDCl_3$) 8.5, 9.6, 86.5, 125.7, 145.7, 152.7, 169.0; *m*/*z* (EI) 204 (M^+ + 2, 100%), 202 (M^+,

100), 191(10), 189 (10), 176 (6), 174 (6), 122 (33), 120 (33), 111 (17), 67 (68); HRMS (M^+) calcd. for $C_7H_7O_2Br$ 201.9629, found 201.9634.

(*E*)-3-(Bromomethylene)isobenzofuran-1(3*H*)-one (4a). Eluent EtOAc–hexane 1 : 6 R_f 0.23; mp 80–85 °C; δ_H (200 MHz; $CDCl_3$) 6.56 (1H, s, =CHBr), 7.62–8.46 (Ar); δ_C (75 MHz; $CDCl_3$) 91.05, 124.56,125.73, 125.97, 131.05, 134.70, 137.23, 147.49, 165.65; *m*/*z* (EI) 226 (M^+ + 2, 98%), 224 (M^+, 100), 170 (11), 168 (11), 104 (48), 89 (82), 76 (52); HRMS (M^+) calcd. for $C_9H_5O_2Br$: 223.9472 found 223.9475.

(*Z*)-3-(Bromomethylene)isobenzofuran-1(3*H*)-one (5a).[15] Eluent EtOAc–hexane 1 : 6 R_f 0.15; mp 128–131 °C; δ_H (200 MHz; $CDCl_3$) 6.33 (1H, s, =CHBr), 7.57–7.91 (Ar); δ_C (75 MHz; $CDCl_3$) 85.72, 119.96, 124.47, 125.84, 130.56, 134.87, 137.90, 148.72, 165.54; *m*/*z* (EI) 226 (M^+ + 2, 98%), 224 (M^+, 100), 170 (10), 168 (10), 104 (36), 89 (83), 76 (41); HRMS (EI) calcd. for $C_9H_5O_2Br$: 223.9472, found 223.9476.

(*E*)- and (*Z*)-3-(Bromomethylene)-4-nitroisobenzofuran-1(3*H*)-one (4b and 5b). Eluent EtOAc–hexane 2 : 3 R_f 0.35 gave a 1 : 1 mixture of **4b** and **5b**; δ_H (200 MHz; $CDCl_3$) 6.95 (1H, s, =CHBr), 7.59 (1H, s, =CHBr), 7.80–8.50 (2 × 3H, m, Ph); *m*/*z* (EI): 271 (M^+ + 2, 23%), 269 (M^+, 23), 218 (34), 190 (49), 161 (55), 104 (60), 75 (100), 57 (59).

(*Z*)-3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one (7).[23] Zinc chloride (3.0 mL, 1.5 mmol, 0.50 M in THF) was added dropwise to a solution of *n*-butyllithium (0.15 mL, 1.5 mmol, 10 M) at −78 °C under N_2. After 1 h a solution of (*Z*)-5-(bromomethylene)-3,4-dimetylfuran-2(5*H*)-one (**3e**) (0.102 g, 0.5 mmol) in dry THF (5 mL) was added, followed by tetrakis(triphenylphosphine)palladium [generated *in situ* from tris(dibenzylideneacetone)dipalladium chloroform adduct (0.026 g, 0.025 mmol) and triphenylphosphine (0.026 g, 0.1 mmol)] in dry THF (4 mL). The mixture was stirred at room temperature for 18 h before the solvent was evaporated off. The residue was dissolved in diethyl ether, washed with a saturated solution of NH_4Cl (10 mL) and brine. The solution was dried ($MgSO_4$), evaporated and the crude product purified by flash chromatography on silica gel. Eluent 0–15% EtOAc in hexane; 0.61 g (68%) yellow oil; δ_H (200 MHz; $CDCl_3$) 0.88 (3H, t, J = 7.0 Hz, $-CH_3$), 1.30–1.42 (2 × 2H, m, 2 × CH_2), 1.86 (3H, s, 4-Me), 1.99 (3H, s, 3-Me), 2.32 (2H, q, J = 7.3 Hz, $-CH_2$), 5 .17 (1H, t, J = 7.8 Hz, CH*n*Bu); δ_C (75 MHz; $CDCl_3$) 8.48, 9.77, 13.74, 22.28, 25.60, 31.26, 110.86, 123.89, 146.83, 149.93, 170.93; *m*/*z* (EI): 180 (M^+, 38%), 137 (98), 138 (16), 125 (27), 124 (100), 110 (16), 82 (17), 55 (65). HRMS (EI) calcd. for $C_{11}H_{16}O_2$: 180.1150, found 180.1147.

Interference with microbial communication

Bioluminescence assay. The biofilm producing type strain *Staphylococcus epidermides* ATCC35984, the AI-2 QS signal producer *Vibrio harveyi* BB152, and the AI-2 QS signal reporter *Vibrio harveyi* BB170 (the *Vibrio*s were kind gifts from Prof. B. L. Bassler) were included in the study. Growth conditions for *V. harveyi* were as described previously.[6,26,30] The second overnight culture of the reporter strain *V. harveyi* BB170 was diluted 1 : 500 in fresh BA-medium and then stored at −70 °C until use.[31] *S. epidermidis* was grown on Brain Heart Infusion agar plates (BHI, Difco Laboratories, Detroit, MI, USA) for 24 h at 37 °C in an aerobic atmosphere before inoculation into Brain Heart Infusion medium (BHI, Difco Laboratories, USA) for biofilm formation and planktonic growth.

A slightly modified bioluminescence assay was performed as previously described[6,26,30] to assess the ability of the various furanones (**2e**, **3b–e**, **4a**, **5a**) to interfere with QS communication. Furanone **F202** was included as a reference. Briefly, cell-free supernatants prepared from *V. harveyi* BB152 were added at final concentration of 10% to BB170. The various furanones were added to a final concentration of 6.0 μmol L^{-1} where upon bioluminescence induction was followed during the next six hours in a Synergy HT Multi-Detection Microplate Reader (Biotek, VT, USA).

Biofilm assay. Biofilm by *S. epidermidis* was allowed to form during 24 h at 37 °C in an aerobic atmosphere on polystyrene discs (Nunc) coated with furanone. Coating with furanone was performed by adding 1 ml 60 μmol L^{-1} furanone dissolved in ethanol to each well. After 24 h, the solvent had evaporated. Discs similarly treated but without furanone were included as controls with no inhibitory effect on biofilm.

Biofilm mass formed was quantified as follows; the discs were rinsed twice in distilled water and stained for 10 min with a 0.1% solution of safranin in new wells. The discs were rinsed again, and bound safranin was released from stained cells using 30% glacial acetic acid. OD measurements at 530 nm were compared to the non-furanone coated control and the reference **F202**.

To exclude an antimicrobial effect of the furanones, total growth, including both scraped biofilm and planktonic cells, was measured. After vigorous shaking to evenly disperse the cells, total growth was quantified by measuring optical density at 600 nm.

Statistics. Each assay was performed in triplicate in three independent experiments. One-way ANOVA on Ranks followed by Student–Newman–Keuls method for normal distribution was used for multiple comparisons on biofilm formation and growth and for comparisons of bioluminescence induction. The level of significance was set at $P \leq 0.01$.

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